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Ruggedness testing and validation of a practical analytical method for >100 veterinary drug residues in bovine muscle by ultrahigh performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

In this study, optimization, extension, and validation of a streamlined, qualitative and quantitative multiclass, multiresidue method was conducted to monitor >100 veterinary drug residues in meat using ultrahigh-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS). Optimization centered on extensive ruggedness evaluation of the method. Various clean-up sorbents were tested and the amount of co-extractives were weighed, matrix effects were measured using post-column infusion of representative analytes, the effect of extract dilution before injecting was studied, and analyte recoveries and reproducibilities were determined. In order to extend our previous method, more drug analytes were added that possessed a wider range of chemical properties, and a re-appraisal of different types of C18 in dispersive solid-phase extraction clean-up and mobile phases in UHPLC–MS/MS was done. Ultimately, end-capped C18 and post-column infusion of ammonium formate as an ionization enhancer for the late-eluting anthelmintics were found to give improved qualitative results for greater analytical scope. A multi-day, multi-analyst validation demonstrated that the final method is suitable for screening of 113 analytes, identifying 98 and quantifying (recoveries between 70–120% and RSD < 25%) 87 out of the 127 tested drugs at or below US regulatory tolerance levels in bovine muscle.

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1. Introduction

Veterinary drugs are widely used for animal food production to prevent diseases or as growth promoters. An improper use may result in drug residue violations in edible tissues, which may cause a risk to human health, so regulation of drugs for use in food animal production is imposed in nearly every country, each of which has somewhat different regulations. For example, the European Union and Canada set "maximum residue levels" (MRLs) of drug residues in the food (or target tissue), and in the US, these are called "tolerances." Even though the animal metabolism and human health risks are not greatly different worldwide, the MRLs/tolerances among different countries are not necessarily the same [1–6]. In an effort to help developing countries and/or attempt to resolve international differences, suggested MRLs and guidelines are also developed by the Codex Alimentarius Commission - Committee on Residues of Veterinary Drugs in Foods [2]. In the US program, meat and poultry products are monitored for veterinary drugs and other adulterants by the Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) [3]. We described the current FSIS operations and the need for use of newer analytical methods previously [7].

Antibiotics are the most widely applied type of drugs, but a wide array of other classes are also used and are included in regulatory monitoring programs. Many single analyte residue methods, single-class, multiresidue methods, and multi-class, multiresidue methods (MMMs) have been reported in the literature [8–10]. In the laboratory, the most efficient way to monitor drug residues in food are MMMs because they are designed to detect a large number of drug residues simultaneously. In our previous work designed for bovine kidney [7], we compared 6 MMMs [11–16] and found that our final method, based on a pre-existing method for β-lactams [12,17], was fast, simple, and reliable, and it also gave good overall performance for screening, identification and quantification of 62 veterinary drugs from 8 different classes.

However, our previous MMM comparison and method validation study did not include several other drugs of interest, such

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as anthelmintics/flukicides, thyreostats, and miscellaneous other veterinary drugs. Schneider et al. [18,19] included up to 120 drug analytes in previous evaluations of the original approach. Additionally, a method for flukicides and anthelmintics residues has been developed, extensively validated, and implemented using modification of the QuEChERS (quick, easy, cheap, effective, rugged and safe) sample preparation method [20] followed by analysis using (ultra)high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) in bovine milk and liver [21–23]. Good quantitative results were obtained when ammonium formate was used in the mobile phase, and negative ionization was needed for flukicides using fast polarity switching.

To help ensure good analytical performance and reliability, method validation requires an in-depth study of many factors, including selectivity, linearity, recoveries, repeatability, ruggedness, reproducibility, matrix effects (MEs), and consistencies of retention times (t_R) and MS ion ratios. Method ruggedness is evaluated by analysis of samples over the course of multiple days using different analysts and reagents to compare results and quality control (QC) performance data. Furthermore, the assessment of MEs and its compensation is highly pertinent in residue analysis. Thus, MEs have been studied by several different approaches [24–33], such as: (i) reducing the amount of co-extractives by improving the extraction and clean-up processes; (ii) achieving a selective chromatographic separation of the analytes from matrix components; (iii) using isotopically labeled internal standards; (iv) calibrating using matrix-matched standards; and (v) doing post-column infusion of the standards [31,32]. Each of these approaches has some drawbacks, and ideally, MEs would not occur in the first place. To provide an excellent assessment of MEs vs. t_R, post-column infusion has been demonstrated by Stahnke et al. [31] and Kittlaus et al. [32]. Even though the accumulated data can be overwhelming in routine analysis, assessing MEs using this approach during method development and validation gives more comprehensive knowledge about the extent of MEs depending on different sample preparation and analysis conditions, which is particularly useful for method optimization.

This study arises from the need of FSIS for a reliable and simple MMM with the capability to screen and identify veterinary drug residues for enforcement monitoring [3,7]. Our objective was to further optimize the MMM, evaluate its ruggedness, and validate the method to monitor >100 drug analytes in bovine muscle. The desired goal was to achieve good screening and identification performance for as many drugs as the method allows and to transfer the method to FSIS for regulatory monitoring. The quantitative performance was a secondary consideration, but it was also measured as a possible additional benefit of the MMM.

2. Materials and methods

2.1. Chemicals and reagents

The prioritized drugs chosen for analysis were divided into two sets (see Table 1): Set #1 contained 61 drug analytes as before [7] and Set #2 consisted of 66 veterinary drugs. All standards were stated to be 90–99.9% purity from US Pharmacopeia (Rockville, MD; USA), Sigma (St. Louis, MO; USA), Berlin-Aldershof GmbH (Berlin, Germany), Dr. Ehrenstorfer (Augsburg, Germany), Janssen Animal Health (Beerse, Belguim), QMX Laboratories (Thaxted, UK), Merial (Lyon, France), Hoechst AG (Frankfurt, Germany), Pfizer Animal Health (New York, NY; USA), Fort Dodge Animal Health (Southampton, UK), except for DCCD which was of unknown purity and provided by Pfizer and sulfabromomethazine also of unknown purity synthesized in-house. All extraction reagents were ACS-grade or higher quality. The hexane was saturated in acetonitrile (MeCN) by adding MeCN until it was no longer miscible. Distilled and deionized water of 18.2 MΩ cm was prepared using a Barnstead E-Pure system (Dubuque, IA, USA). Formic acid (88% purity) was from Spectrum (Gardena, CA, USA). Stock and mixed drug solutions for spiking and calibration were prepared in MeCN in glass vials for all drugs except β-lactams, for which water and plastic vials were used. Storage of solutions was at −18 °C in the dark. Sorbents were obtained from multiple sources: bulk C18 (40 μm particles) was from Supelco (Bellefonte, PA, USA), J.T. Baker (Phillipsburg, NJ, USA), and Applied Separations (Allentown, PA, USA). Bulk end-capped C18 was obtained from UCT (Bristol, PA, USA), and Z-Sep and Z-Sep$^+$ were obtained from Supelco. Reagents for UHPLC mobile phase were Optima quality from Fisher (Suwanee, GA, USA), ammonium formate (99% purity) and formic acid (98% purity) was from Sigma.

2.2. Instrumentation

Extracts were shaken by a Glas-Col platform pulse mixer (Terre Haute, IN, USA). A Zymark TurboVap LV system (Hopkinton, MA, USA) and a ThermoFisher Sorvall Legend RT centrifuge (Waltham, MA, USA) were used for solvent evaporation and centrifugation, respectively.

A Waters (Milford, MA, USA) Acquity TQD UHPLC-MS/MS controlled by MassLynx software was used for analysis. Electrospray ionization was employed, and among the 127 drug analytes, only 8 (clorsulon, nitroxynil, triclabendazole sulfoxide, oxyclonazide, niclosamide, bithionol, closantel, rafoxanide) had better performance in negative than positive mode, and as the instrument is capable of fast polarity switching, both ionization modes were used. Optimization of MS/MS parameters (choice of ions, cone voltages, and collision energies) was done by infusion of standards and the use of Intellistart software. Based on optimization results, an acquisition method was created for each set of drug analytes. Minimum dwell time of 5 ms for the instrument was used in all cases to maximize the number of data points per peak. Fixed parameters included 3 kV capillary voltage, 3 V extractor voltage, and 450° and 150 °C desolvation and source temperatures, respectively. Different sets of spray cones for interchangeable usage were cleaned after each sequence.

Vials were maintained at 4 °C in the autosampler tray to reduce possible degradation. For the UHPLC, a Waters Acquity reversed-phase HSS T3, 100 mm long, 2.1 mm i.d., 1.8 μm particle size was used with a mobile phase consisting of 0.1% formic acid in both (A) 5:95 (v/v) MeCN:water and (B) MeCN. The gradient was 99.8% A for 0.1 min, then to 99.8% B until 8 min, followed by 1.5 min hold time. The re-equilibration time to 99.8% A took 3.3 min until the next injection. Column temperature was kept at 40 °C, flow rate was 0.5 mL/min, and injection volume was 20 μL. For analysis of analyte Set #2, 50 μL/min of 27 mM ammonium formate in MeOH:MeCN (75:25) was infused from 5.05 to 9.45 min using the instrument's infusion syringe to enhance the signal of the late-eluting anthelmintics. Table 1 lists the ion transitions and retention times (t_R) among other facets in the UHPLC–MS/MS analysis.

2.3. Sample preparation

FSIS Midwestern Laboratory (St. Louis, MO) provided 44 different bovine muscles from slaughtered animals, which were shipped in dry ice and stored at −18 °C. The 44 bovine muscles were prescreened and the 24 that showed no presumptive positives were used as "matrix blanks" for spiking and other experiments. Each of the 24 frozen muscles were individually processed by dicing 100 g portions using a knife and homogenized with a kitchen food processor using plastic bowls. Also, a mixture of equal parts of the 24 muscles was used in some experiments as an "average" matrix blank. In the method validation experiments, matrix-matched

Table 1
List of drugs chosen for analysis, internal standards (IS), quality control analyte (QC), US tolerances, lowest spiking level (½X, X = targeted level), retention times (t_R) and MS/MS conditions for analysis (CE = collision energy).

Set #	#	Analyte	Drug class	US tol. (μg/g)	½X spike level (μg/g)	t_R (min)	Precursor ion (*m/z*)	Cone (V)	Product ions (*m/z*)					
									Quant ion 1	CE (V)	Qual ion 2	CE (V)	Qual ion 3	CE (V)
1	1	Desacetyl Cephapirin	β-Lactam	0.1[a]	0.05	0.69	382.1	32	152.0	28	124.2	48	111.3	48
1	2	Florfenicol Amine	Phenicol	0.3[b]	0.15	0.68	248.1	25	230.2	10	130.1	35	131.1	20
1	3	Sulfanilamide	Sulfonamide		0.05	1.19	173.0	40	92.9	20	75.9	36	65.9	32
1	4	Amoxicillin	β-Lactam	0.01	0.005	1.47	366.1	20	114.0	22	349.3	10	208.1	14
1	5	Salbutamol	β-Agonist		0.003	1.46	240.2	20	148.2	20	222.3	10	166.2	15
1	6	Zilpaterol	β-Agonist	0.012[c]	0.006	1.46	262.3	27	244.3	12	185.2	30	202.2	21
1	7	Cimaterol	β-Agonist		0.003	1.51	220.0	16	143.0	24	115.9	34	88.9	46
1	8	DCCD[*]	β-Lactam	0.4	0.5	1.72	549.1	40	183.0	30	241.1	20	125.9	76
1	9	Lincomycin	Lincosamide	0.1[d]	0.05	1.87	407.3	20	126.1	30	359.2	20	389.2	18
1	10	Sulfadiazine	Sulfonamide		0.05	2.00	251.1	30	156.1	15	108.0	20	158.1	15
1	11	Ampicillin	β-Lactam	0.01	0.005	2.01	350.1	26	106.1	24	114.0	30	160.1	24
1	12	Desethylene Ciprofloxacin	Fluoroquinolone	0.1[c]	0.025	2.06	306.2	35	288.2	20	245.2	20	289.3	10
1	13	Sulfathiazole	Sulfonamide	0.1[e]	0.05	2.10	256.1	25	156.1	15	108.0	25	101.0	25
1	14	Sulfapyridine	Sulfonamide		0.025	2.18	250.1	32	156.1	18	108.1	28	92.0	26
1	15	Norfloxacin	Fluoroquinolone	[f]	0.05	2.16	320.2	36	276.2	18	233.1	26	219.0	30
1	16	Tulathromycin	Macrolide	5.5[c]	0.05	2.17	806.8	38	72.0	56	577.5	24	116.0	50
1	17	Oxytetracline	Tetracycline	2[g]	0.025	2.21	461.2	25	426.4	20	443.4	15	201.2	35
1	18	Ciprofloxacin	Fluoroquinolone	[f]	0.5	2.22	332.2	35	245.2	25	288.4	20	203.2	40
1	19	Ractopamine	β-Agonist	0.03[h]	0.001	2.27	302.2	26	164.0	16	107.0	32	121.0	24
1	20	Sulfamerazine	Sulfonamide		0.05	2.30	265.1	28	91.9	28	155.9	16	107.9	26
1	21	Danofloxacin	Fluoroquinolone	0.2[c,h]	0.025	2.31	358.1	28	96.0	26	314.2	18	283.1	26
1	22	Tetracyline	Tetracycline	2[g]	0.5	2.35	445.2	30	154.1	30	410.2	20	427.3	15
1	23	Enrofloxacin	Fluoroquinolone	0.1[j]	0.025	2.38	360.2	35	316.4	20	245.3	25	203.2	40
1	24	2-Quinoxalinecarboxylic Acid	Other	[i]	0.015	2.43	175.0	22	129.0	16	131.0	16	102.0	30
1	25	Sulfamethizole	Sulfonamide		0.05	2.55	271.1	28	156.1	16	92.0	30	108.0	26
1	26	Sulfamethazine	Sulfonamide	0.1[a]	0.05	2.54	279.1	35	186.1	20	156.1	20	108.1	25
	27	**Sulfamethazine-^{13}C6 (IS)**			0.4	2.54	285.2	32	186.1	18	124.1	26	98.0	32
1	28	Cefazolin	Cephalosporin		0.05	2.56	455.1	20	156.0	16	323.2	12	112.1	35
1	29	Sulfamethoxypyridazine	Sulfonamide		0.05	2.58	281.1	30	156.1	20	126.2	20	108.1	25
1	30	Difloxacin	β-Lactam		0.025	2.62	400.3	35	356.4	20	299.2	30	285.3	40
1	31	Sarafloxacin	Fluoroquinolone	[f]	0.025	2.58	386.1	20	342.2	20	299.2	30	270.2	48
1	32	Clenbuterol	β-Agonist	[f]	0.003	2.56	277.2	25	259.2	10	132.1	30	203.1	15
1	33	Pirlimycin	Lincosamide	0.3[h]	0.15	2.74	411.3	30	112.2	40	363.3	20	110.3	45
1	34	Chlortetracycline	Tetracycline	2[g]	1	2.84	479.2	30	154.1	30	444.3	20	462.2	20
1	35	Clindamycin	Lincosamide		0.05	2.89	425.3	45	126.2	40	377.4	20	124.3	45
1	36	Gamithromycin	Macrolide		0.05	2.91	777.8	62	83.0	54	116.0	50	158.1	46
1	37	Sulfachloropyridazine	Sulfonamide	0.1[a]	0.05	2.95	285.0	28	156.1	16	108.0	26	92.0	30
1	38	Tilmicosin	Macrolide	0.1[h]	0.05	3.06	869.8	45	174.2	35	696.6	35	132.2	35
1	39	Sulfadoxine	Sulfonamide		0.05	3.10	311.2	35	156.1	20	108.1	30	140.1	30
1	40	Sulfamethoxazole	Sulfonamide		0.05	3.11	254.0	26	92.1	30	156.0	18	107.9	28
1	41	Sulfaethoxypyridazine	Sulfonamide	0.1[a]	0.05	3.14	295.1	30	156.1	20	140.2	20	108.1	25
1	42	Florfenicol	Phenicol	0.2[d]	0.1	3.15	358.1	24	241.0	18	206.0	28	130.4	50
1	43	Chloramphenicol	Phenicol	[f]	0.002	3.36	323.1	16	275.0	16	165.0	26	118.8	42
1	44	Erythromycin	Macrolide	0.1[a]	0.05	3.49	734.8	30	158.2	36	115.9	54	576.5	20
1	45	Sulfadimethoxine	Sulfonamide	0.1[a]	0.05	3.57	311.1	35	156.1	20	108.0	30	245.2	20
1	46	Sulfaquinoxaline	Sulfonamide	0.1[a]	0.05	3.59	301.1	34	156.1	18	108.0	28	92.0	36
1	47	Prednisone	Corticosteroid		0.05	3.67	359.2	22	341.1	10	146.9	26	267.3	15
1	48	Tylosin	Macrolide	0.2[k]	0.1	3.66	916.8	45	174.2	35	101.1	35	145.2	35
	49	**Penicillin G-d7 (IS)**			0.4	3.86	342.1	46	183.1	26	160.1	24	98.1	54
1	50	Penicillin G	β-Lactam	0.05[a]	0.025	3.86	335.1	18	176.0	16	160.1	18	114.0	30
1	51	Beta/Dexa-methasone	Corticosteroid		0.05	4.11	393.2	20	373.2	10	147.1	28	355.3	14
1	52	Sulfanitran	Sulfonamide		0.05	4.16	336.2	26	156.0	12	134.1	28	92.7	36
1	53	Sulfabromomethazine	Sulfonamide	0.1[a]	0.05	4.21	357.1	35	92.0	30	156.1	25	108.1	35

Table 1 (*Continued*)

Set #	#	Analyte	Drug class	US tol. (μg/g)	½X spike level (μg/g)	t_R (min)	Precursor ion (*m/z*)	Cone (V)	Product ions (*m/z*)					
									Quant ion 1	CE (V)	Qual ion 2	CE (V)	Qual ion 3	CE (V)
1	54	Zeranol (α-Zearalanol)	Other	[l]	0.003	4.37	323.2	16	305.2	10	189.1	24	149.0	30
1	55	Oxacillin	β-Lactam		0.05	4.39	402.1	22	160.0	20	243.1	18	144.1	34
	56	**Atrazine (QC)**			0.4	4.49	216.1	34	174.0	18	103.9	30	95.9	26
1	57	Cloxacillin	β-Lactam	0.01[a]	0.005	4.66	436.2	22	160.1	12	277.1	16	114.1	44
1	58	Nafcillin	β-Lactam		0.05	4.79	415.2	20	199.1	14	171.1	38	115.1	78
1	59	Oxyphenylbutazone	NSAID		0.05	4.83	325.2	26	120.1	24	148.2	30	204.1	16
1	60	Flunixin	NSAID	0.025[h]	0.013	4.86	297.1	42	279.1	22	109.0	50	264.0	32
	61	**Flunixin-d3 (IS)**			0.4	4.82	300.1	40	282.1	24	112.0	54	264.0	36
1	62	Dicloxacillin	β-Lactam		0.05	5.03	470.2	22	160.1	14	311.1	16	114.1	48
1	63	Phenylbutazone	NSAID		0.05	5.93	309.1	28	120.0	20	91.8	30	76.9	50
1	64	Melengesterol Acetate	Other	0.025[m]	0.01	6.30	397.4	30	279.3	20	337.5	15	221.3	40
2	65	2-Thiouracil	Thyreostat		0.2	0.85	128.9	32	111.9	12	69.9	18	83.9	22
2	66	2-Mercapto-1-methylimidazole	Thyreostat		0.1	1.14	114.9	40	87.9	16	73.9	16	82.7	16
2	67	6-Methyl-2-thiouracil	Thyreostat		0.2	1.22	142.9	32	83.9	18	125.9	14	66.9	26
2	68	Metronidazole-OH	Nitroimidazole	[f]	0.005	1.42	188	22	123	14	126	18	143.9	14
2	69	Dipyrone	Tranquilizer		0.1	1.60	218.1	24	96.9	12	187	10	125	12
2	70	Dimetridazole-OH	Nitroimidazole	[f]	0.025	1.63	158	22	140	12	93.9	22	111.9	20
2	71	Metronidazole	Nitroimidazole	[f]	0.005	1.63	172	26	127.9	14	81.9	24	110.9	24
2	72	5-Hydroxythiabendazole	Anthelmintic		0.05	1.70	218	50	190.9	26	147	32	119.7	42
2	73	Albendazole 2-amino-sulfone	Anthelmintic	0.05[h,n]	0.025	1.85	240	36	133	28	198	20	105.6	46
2	74	Ronidazole	Nitroimidazole	[f]	0.005	1.85	201	18	139.9	10	54.8	20	66.9	32
2	75	Levamisole	Anthelmintic	0.1[h]	0.05	1.86	205	40	178	22	90.9	34	122.9	28
2	76	Dimetridazole	Nitroimidazole	[f]	0.005	1.86	142	32	95.9	16	80.9	24	111.9	12
2	77	Thiabendazole	Anthelmintic	0.1[h]	0.05	1.94	202	44	174.9	26	130.9	32	64.9	40
2	78	6-propyl-2-thiouracil	Thyreostat		0.025	2.15	171	38	154	18	112	20	66.9	26
2	79	2-Mercaptobenzimidazole	Thyreostat		0.0125	2.30	150.9	42	92.8	20	118	22	64.9	30
2	80	Azaperone	Tranquilizer		0.005	2.34	328.3	34	165	20	122.9	36	94.9	62
2	81	Orbifloxacin	Fluoroquinolone	[f]	0.025	2.39	396.2	36	352.2	18	295.1	24	378.2	20
2	82	Albendazole sulfoxide	Anthelmintic		0.025	2.44	282.1	28	240	14	207.3	24	159	38
2	83	Xylazine	Tranquilizer		0.005	2.48	221.1	42	164	26	147	24	105	36
2	84	Ipronidazole-OH	Nitroimidazole	[f]	0.003	2.54	186.1	22	168	14	121.8	20	127.9	16
2	85	Morantel	Anthelmintic		0.05	2.60	221.1	50	122.9	36	163.9	28	110.9	24
2	86	2-Amino-Mebendazole	Anthelmintic		0.005	2.63	238.1	50	104.9	26	132.9	36	90.9	46
2	87	6-Phenyl-2-thiouracil	Thyreostat		0.2	2.73	205	38	103	26	187.9	18	146	20
2	88	2-Amino-Flubendazole	Anthelmintic		0.005	2.77	256	50	122.9	28	94.9	38	132.9	36
2	89	Cambendazole	Anthelmintic		0.005	2.83	303.1	34	261.1	18	217	28	190	40
2	90	Bacitracin	Other	0.5[a]	0.25	2.87	475.3	26	85.9	24	199.1	30	110	54
2	91	Carazolol	Tranquilizer		0.005	2.90	299.3	34	116	20	97.9	22	222	22
2	92	Doxycycline	Tetracycline		0.05	2.91	445.3	28	428.2	20	97.9	46	153.9	32
2	93	Oxibendazole	Anthelmintic		0.005	2.95	250.1	34	218.1	18	175.9	28	147.9	36
2	94	Oxfendazole	Anthelmintic	0.8[h]	0.013	3.01	316.1	40	158.9	32	191	22	284	18
2	95	Albendazole sulfone	Anthelmintic		0.025	3.02	298.1	38	266	20	159	36	223.9	26
2	96	Ipronidazole	Nitroimidazole	[f]	0.001	3.20	170.1	34	124	18	109	24	95.8	22
2	97	Clorsulon	Flukicide	0.1[h]	0.05	3.39	377.7	24	341.8	12	241.9	20	141.9	28
2	98	Haloperidol	Tranquilizer		0.005	3.53	376.2	40	165	24	122.9	42	94.9	68
2	99	Acetopromazine	Tranquilizer		0.005	3.55	327.2	32	86	20	254	22	222.1	42
2	100	Promethazine	Tranquilizer		0.005	3.58	285.2	24	85.9	16	198	20	240.1	16
2	101	Fenbendazole sulfone	Anthelmintic	2[p]	0.5	3.65	332.1	40	300	22	158.9	38	130.9	50
2	102	Albendazole	Anthelmintic		0.025	3.65	266.1	34	234	20	191.1	32	158.9	38
2	103	Mebendazole	Anthelmintic		0.005	3.70	296.1	36	264.1	20	104.9	36	158.8	46
2	104	Flubendazole	Anthelmintic		0.005	3.90	314.1	38	282	22	94.9	50	122.9	38
2	105	Propionylpromazine	Tranquilizer		0.005	3.91	341.2	32	85.9	22	268.1	24	236	40
2	106	Chlorpromazine	Tranquilizer		0.005	4.04	319.2	32	86	20	246	22	213.9	42
2	107	Triflupromazine	Tranquilizer		0.005	4.26	353.2	34	85.9	22	280	28	248	44

Table 1 (*Continued*)

Set #	#	Analyte	Drug class	US tol. (μg/g)	½X spike level (μg/g)	t_R (min)	Precursor ion (*m/z*)	Cone (V)	Product ions (*m/z*)					
									Quant ion 1	CE (V)	Qual ion 2	CE (V)	Qual ion 3	CE (V)
2	108	Fenbendazole	Anthelmintic	0.4[h,o], 2[d]	0.2	4.33	300.1	38	268	20	158.9	36	130.9	46
2	109	Oleandomycin triacetate	Macrolid	0.15[e]	0.075	4.37	814.7	38	200.1	30	98	48	84.9	80
2	110	Nitroxynil	Flukicide		0.025	4.41	288.8	40	126.8	20	115.9	34	89	32
2	111	Virginiamycin M1	Other	0.1[d]	0.05	4.49	526.4	26	508.3	12	108.9	44	355.1	18
2	112	Ketoprofen	Tranquilizer		0.005	4.71	255.1	28	104.9	24	209	14	76.8	40
2	113	Haloxon	Anthelmintic	0.1[a]	0.05	5.28	415.1	44	272.9	34	210.9	36	353	20
2	114	Triclabendazole sulfoxide	Flukicide		0.025	5.37	372.8	36	357.8	18	212.9	30	180.8	44
2	115	Emamectin benzoate	Anthelmintic		0.005	5.49	886.8	52	158	40	126	46	302.2	28
2	116	Diclofenac	Tranquilizer		0.1	5.55	296	20	214.9	20	250	12	278	10
2	117	Triclabendazole	Flukicide		0.025	5.99	359	52	343.9	26	274	38	170.9	56
2	118	Novobiocin	Other	1[a]	0.5	6.05	613.5	30	189	28	132.9	64	218	14
2	119	Oxyclozanide	Flukicide		0.005	6.08	399.6	38	363.8	14	175.9	24	201.8	24
2	120	Niclosamide	Flukicide		0.005	6.20	325	36	170.9	30	289	16	134.9	46
2	121	Tolfenamic acid	Tranquilizer		0.1	6.23	262.1	22	244	14	180	40	228.9	32
2	122	Bithionol	Flukicide		0.005	6.76	352.9	36	160.8	24	191.2	28	124.2	44
2	123	Eprinomectin	Anthelmintic	0.1[h]	0.05	7.44	914.8	18	186.1	20	154	40	112	74
2	124	Abamectin	Anthelmintic		0.025	7.94	890.8	16	305.3	28	145	42	113	46
2	125	Closantel	Flukicide		0.025	8.07	660.9	70	126.8	54	344.8	32	314.9	34
2	126	Doramectin	Anthelmintic	0.03[h]	0.05	8.30	916.9	22	331.3	26	113	56	145	48
2	127	Moxidectin	Anthelmintic	0.05[h,n]	0.025	8.32	640.5	16	528.3	8	498.2	10	199	22
2	128	Rafoxanide	Flukicide		0.005	8.50	623.9	62	126.1	48	344.8	30	140.9	54
2	129	Selamectin	Anthelmintic		0.1	8.62	770.7	36	145	30	112.9	40	94.9	52
2	130	Ivermectin Na+	Anthelmintic	0.01[h], 0.02[d]	0.05	8.77	897.8	82	183	58	329.2	56	240.1	64

[*] Desfuroylceftiofur cysteine disulfide (DCCD) is a marker residue metabolite of ceftiofur.
[a] Tolerance in uncooked edible tissue of cattle.
[b] Marker residue for florfenicol in cattle muscle.
[c] Tolerance in cattle liver, (for tulathromycin, a marker residue has been established).
[d] Tolerance in swine muscle.
[e] Tolerance in uncooked edible tissue of swine.
[f] Banned for extralabel use.
[g] Tolerance is for the sum of residues of tetracycline including chlortetracycline, oxytetracycline, and tetracycline in muscle.
[h] Tolerance in cattle muscle.
[i] Metabolite of carbadox, which has a tolerance in swine liver of 0.03 μg/g.
[j] Tolerance is for desethylene ciprofloxacin (marker residue) in cattle liver.
[k] Tolerance in uncooked cattle fat, muscle, liver, and kidney.
[l] A tolerance is not needed for cattle.
[m] Tolerance in cattle fat.
[n] Tolerance in sheep muscle.
[o] Tolerance in goat muscle.
[p] Tolerance in turkey muscle. Tolerances obtained from the FDA Code of Federal Regulations. July, 2012. (http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?CFRPart=556).

calibration standards and "mixed muscle" replicates (4 of the 10 spikes at each level) were prepared from a mixture of the 6 different beef samples used in each day's experiment.

The final sample preparation method was similar to ones previously reported [7,12,18] with the following steps: (1) add 10 mL of MeCN/water (4/1, v/v) to 2 g homogenized sample in 50 mL polypropylene (PP) tubes; (2) shake for 5 min at the 70% setting with full pulsation; (3) centrifuge at 25 °C for 5 min at 3716 rcf (max centrifuge setting); (4) transfer the extract to a 50 mL graduated PP centrifuge tube containing 500 mg of end-capped C18 sorbent; (5) add 10 mL hexane pre-saturated with MeCN; (6) shake for 30 s; (7) centrifuge again as in step 3; (8) aspirate hexane to waste; (9) evaporate 5 mL of the extract under nitrogen flow at 45 °C to <0.7 mL; (10) add water with 0.1% formic acid to reach the 1 mL mark (1 g/mL sample equivalent); and (11) filter 0.5 mL of the final extracts using 0.2 μm PVDF filter vials from Thomson Instrument Co. (Oceanside, CA). Different sorbents with and without the hexane additions were used in method optimization experiments as described later.

For matrix-matched calibration, the spiking and internal standard (IS) solutions were added to the blank extracts during step 10 above to yield the desired equivalent analyte concentrations in the samples (at ¼X, ½X, 1X, 2X and 3X levels, where X corresponds to the targeted level, as given in Table 1). A quality control (QC) spike of atrazine (a triazine herbicide) at 400 ng/g was also added to all extracts and standards during Step 11 to evaluate instrument performance. Care was taken in the additions for sample extracts and calibration standards alike to reasonably match the composition of MeCN in all injected samples.

2.4. Amount of co-extractives

The amount of co-extractives was determined by gravimetric measurements [33] for 9 different clean-up conditions: hexane only; C18 only; Z-Sep$^+$ only; Z-Sep only; Z-Sep + C18; C18 + hexane; Z-Sep$^+$ + hexane; Z-Sep + hexane; and Z-Sep + C18 + hexane. Stepwise, this involved: (1) heating glass test tubes for 1 h at 110 °C to remove moisture; (2) pre-weighing test tubes on a calibrated analytical balance; (3) transferring a 5 mL aliquot of the initial blank extract and the cleaned-up blank extracts, each in duplicate; (4) taking all extracts to dryness on the TurboVap at 60 °C; (5) heating the tubes at 110 °C for 1 h to remove moisture; (6) re-weighing the test tubes on a calibrated analytical balance and recording the differences. The amount of co-extracted matrix was the average difference between the initial and post-extraction weights of the same tube, and the amount removed by cleanup was the average weight difference of the matrix components before and after cleanup.

2.5. Matrix effect assessment

After taking into account the gravimetric results, MEs in UHPLC–MS/MS were evaluated by injections of mixed matrix blanks obtained using the 3 most promising cleanup variations (Z-Sep$^+$, C18 + hexane, and Z-Sep + hexane). In an approach similar to previous reports [31,32], sample injections were made while simultaneous post-column infusion was conducted at 20 μL/min of a mixture of 15 representative analytes at 400 ng/mL (sulfamethoxypyridazine, ciprofloxacin, prednisone, zilpaterol, nafcillin, acepromazine, fenbendazole, levamisole, 2-amino-mebendazole, bithionol, clorsulon, niclosamide, nitroxynil, rafoxanide, triclabendazole sulfoxide). These analytes were chosen based in their physicochemical properties, retention times, and ionization characteristics. For the samples that underwent cleanup with C18 + hexane, the MEs for 2- and 4-fold dilutions were also measured by the same approach.

Two transitions were monitored for each analyte. The data acquired was smoothed using MassLynx software Smooth Tool by setting a window size of ±5 scans, smooth factor of 2, and "mean" as the smoothing method. From the 2106 smoothed data points obtained during the chromatogram, MEs for each analyte were calculated as the %differences between the signals obtained when matrix extracts were injected and when solvent-only (H_2O–MeCN 95:5, 0.1% formic acid) was injected, divided by the signal of the latter. An Excel spreadsheet was used for calculations and plots.

To check for the possibility of ghost peaks which would weaken the quality of the experiment, we looked for matrix components in subsequent injections. No carry-over was observed beyond 3 solvent-only injections between samples; thus, 3 solvent-only injections were made between each sample in this experiment. The sequence was set to inject the sample extracts in the following order: C18 + hexane, Z-Sep$^+$, Z-Sep + hexane and raw extracts.

2.6. Method optimization and validation

For the 3 most promising clean-up variations (Z-Sep$^+$, C18 + hexane, and Z-Sep + hexane), recoveries (Rec) and repeatabilities (RSD_r) were calculated for samples spiked at 2X (see Table 1) in the case of Set #1 and 200 ng/g for Set #2, each in triplicate. Two sets of samples ($n = 3$) were spiked with standard solution Set #1 and Set #2, respectively. Additionally, possible loss of analytes in the hexane phase was checked.

Validation of the final optimized method was done separately for each set of analytes and muscle samples on different days by different analysts using different pipettes and reagents. Twenty-four different bovine muscle samples (plus a mixture of them all) were analyzed in the course of 4 days by 3 different chemists using C18 from 4 different vendors. On one day, 4-fold higher concentrations of the β-lactams/cephalosporins were accidentally spiked. In the case of Set #2, there was a considerable effect of end-capped C18 on recoveries, and two more days of validation were conducted. Only the data from 3 of the days using end-capped C18 was included in the quantification assessment, but all data was used for screening and identification purposes. Ten replicates each at 3 spiking levels for 6 different muscle samples (including 4 replicates at each level for the combined samples) were analyzed each day. Spikes were made at ½X, 1X, and 2X concentrations as shown in Table 1. Also, 2 isotopically labeled IS, sulfamethazine-$^{13}C_6$ and flunixin-d_3, were added prior to extraction along with the drug analytes in spiked samples. In analytical sequences, reagent-only and matrix-matched calibration standards were interspersed among spiked samples of the same expected concentrations. After the 3X standard, two reagent blank injections were made to check for possible carry-over.

3. Results and discussion

3.1. Choice of drug analytes and target levels in bovine muscle

In our previous work [7], we chose the targeted concentrations of the 61 priority drug analytes of concern in bovine kidney based on US tolerance levels, international MRLs, and detection limits from other methods used by FSIS [34]. Many of the tolerances for the same drugs are different in muscle than kidney, thus spiking concentrations had to be adjusted accordingly (see Table 1). Furthermore, we decided to extend the method to an additional 66 drugs from other classes, which required us to look up their tolerances, MRLs, and detection needs in muscle as was done for the 61 drugs in kidney previously. By extending the MMM to these additional analytes and muscle, FSIS would gain analytical efficiency as well as flexibility to repurpose existing residue methods.

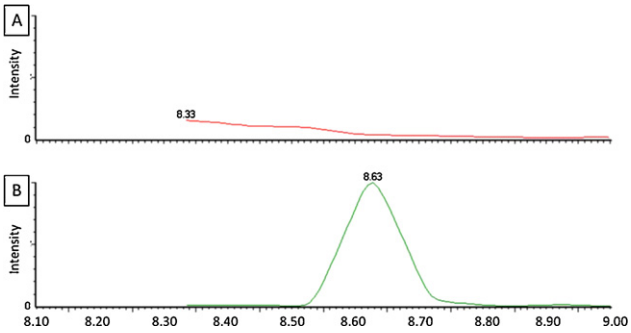

Fig. 1. Signal enhancement of selamectin using post-column infusion of 27 mM ammonium formate in MeOH:MeCN (75:25) at 50 μL/min: (A) 200 ng/g concentration without infusion; (B) 100 ng/g equivalent sample concentration with infusion.

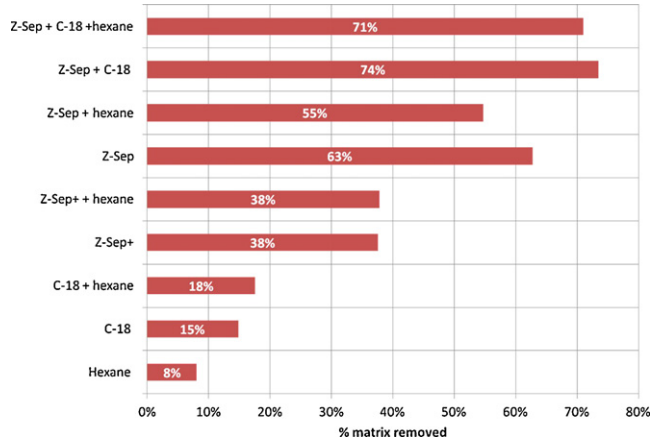

Fig. 2. Average percent of matrix removed after cleanup when using different sorbents in d-SPE and/or partitioning with hexane in bovine muscle ($n = 2$). The initial extract contained 2.9% matrix co-extractives from the original sample.

3.2. Post-column infusion for anthelmintics

In any method development study, analytical parameters for detection must be optimized prior to studying sample preparation conditions. In this study, we originally tried to simply add 67 more drug analytes to the pre-existing UHPLC method [7]. All analytes were infused at concentration of 1 μg/mL and tuned using the instrument's signal optimization software. In accordance with our mobile phase, drug standards were originally infused using formic acid as the additive. However, Kinsella et al. [21] demonstrated that anthelmintics gave better results when ammonium salts were used in the mobile phase. Therefore, ammonium formate was also tested for determining MS/MS detection conditions, and as reported previously [21], the signals for the anthelmintics increased dramatically. Afterwards, a mobile phase consisting of (A) 5 mM ammonium formate in H_2O:MeOH:MeCN (95:3.5:1.5) and (B) 5 mM ammonium formate in MeOH:MeCN (75:25) was tested for all 127 drugs in sample analyses, but strange results were observed for a few β-agonists, sulfas, and others, presumably due to enhanced ionization of matrix components toward the front of the chromatogram. Because the improvements for the anthelmintics were based on ionization efficiency, not chromatography, and since these analytes had t_R between 5 and 9 min, we decided to use post-column infusion of ammonium formate in MeOH:MeCN (75:25) when anthelmintics eluted at the end of the chromatographic run. This approach only potentially affected the ionization of phenylbutazone and melengestrol acetate from Set #1, and their results gave no significant differences with and without the use of infusion. Fig. 1 demonstrates the dramatic signal intensity improvement of selamectin by using the post-column infusion of ammonium formate.

3.3. Amount of co-extractives

The amount of co-extractives was calculated for 9 different combinations of dispersive solid phase extraction (d-SPE) cleanup and/or partitioning with hexane. The amount of co-extractives in initial extracts of the mixed muscle matrix was 2.9% (58 mg in a 2 g sample). Fig. 2 shows the % removal for each cleanup tested. Liquid–liquid extraction with hexane was the least efficient cleanup followed by C18 and the combination of C18 + hexane, which achieved a total of 18% removal of matrix components by weight. Z-Sep$^+$ and Z-Sep gave lower amounts of co-extractives, removing 38% and 63% of matrix, respectively. When combinations of Z-Sep$^+$ and Z-Sep with hexane were tested, no additional removal was observed, and perhaps the solvent made cleanup efficiency even worse, as noticed in the 8% reduction in removal efficiency for Z-Sep + hexane. Ultimately, the combination of Z-Sep and C18 resulted in the highest matrix removal of 74%. Despite the better cleanup efficiency by weight provided by these novel sorbents, further experiments that measured MEs and recoveries did not show the same benefits, as discussed in the following sections.

3.4. Matrix effects

Post-column infusion of representative analytes was used for evaluation of MEs in the raw extracts and 3 extracts cleaned-up with Z-Sep$^+$, C18 + hexane, and Z-Sep + hexane. One analyte from each main chemical group was chosen to provide a broad set of physicochemical properties. For example, Fig. 3 shows the ME profiles for the C18 + hexane extract for sulfamethoxypyridazine ($pK_a = 6.7$, $\log K_{ow} = 0.32$ and surface tension = 75.1 mN/m) nafcillin ($pK_a = 2.6$, $\log K_{ow} = 3.79$ and surface tension = 70 mN/m), acepromazine ($pK_a = 9.3$, $\log K_{ow} = 4.24$ and surface tension = 45 mN/m), and levamisole ($pK_a = 8.0$, $\log K_{ow} = 1.84$ and surface tension = 54.1 mN/m) [35]. This experiment demonstrated that even though the factors that affect the ionization process are variable, the MEs were similar for all analytes tested in ESI+ mode. When negative mode was used, the ME profiles did not follow a clear trend. These observations are in accordance with Stahnke et al. [31] and Kittlaus et al. [32]. Nonetheless, the 6 drugs ionized in negative mode showed signal enhancement with t_R between 8 and 10 min. A reduction of these MEs was observed when Z-Sep$^+$ or C18 + hexane were used.

Because all analytes ionized in positive mode behaved similarly, only the ME profiles for fenbendazole obtained using different cleanup sorbents are shown in Fig. 4. An intense ion suppression

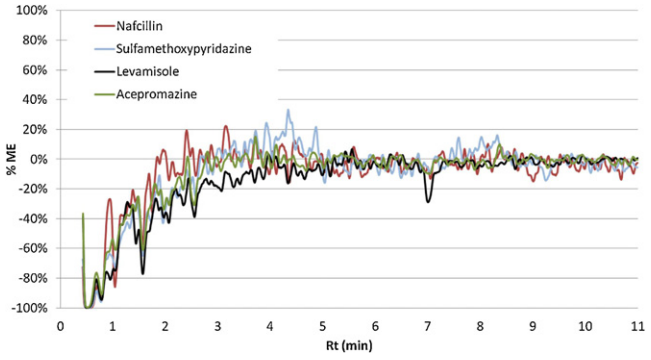

Fig. 3. Matrix effect (ME) profiles obtained by post-column infusion of sulfamethoxypyridazine, nafcillin, acepromazine, and levamisole in an extract cleaned-up with C18 + hexane.

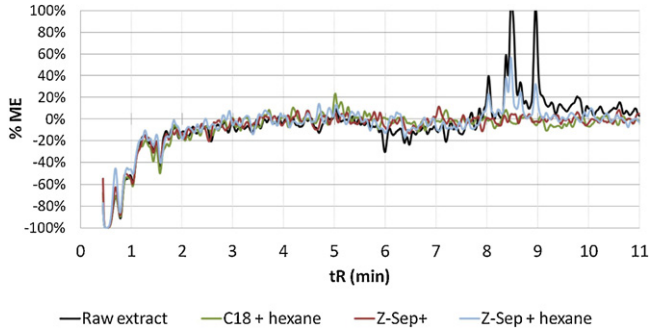

Fig. 4. Matrix effect (ME) profiles obtained by post-column infusion of fenbendazole (m/z 300 → m/z 268) in 4 extracts obtained using different clean-up sorbents; the veterinary drug analytes elute between 0.7 and 10 min.

effect (ME < −60%) was observed for all extracts between 0.4 and 1.0 min, which explains the poor results for the 3 early-eluting analytes (desacetyl cephapirin, florfenicol amine and 2-thiouracil), as also noted previously [7,19]. In the case of raw extracts, no significant MEs were observed between 1.7 and 8 min, but an enhancement effect was observed between 8 and 9 min when non-polar matrix compounds co-elute. This enhancement was similar to the observed results in negative mode. Z-Sep$^+$ and C18 + hexane gave almost no MEs between 1.7 and 11 min, showing a significant reduction of MEs in comparison with the raw extracts. Nevertheless, even for Z-Sep$^+$ and C18 + hexane co-elution of polar matrix compounds were still problematic at the beginning of the run. In the case of Z-Sep + hexane, the profile was similar to the raw extracts, which gave ME > 20% from 7.8 to 9.2 min. This is very interesting considering that although Z-Sep + hexane demonstrated high removal efficiency by weight, the components extracted were not the ones causing the enhancement effects. In an injection of reagent blank in which the 2 g sample was replaced by water during extraction, no direct interferences from the extraction solvent and cleanup reagents were observed in any analyte ion transition tested.

3.5. Dilution of extracts

In an attempt to reduce MEs and improve method ruggedness, a 2- and 4-fold dilution of the final extracts obtained using C18 + hexane as cleanup was evaluated. When a 2-fold dilution was done, reduction in MEs was observed after 2 min in the chromatogram. However, a 4-fold dilution was necessary to reduce the intense suppression between 1 and 2 min, as shown in Fig. 5. Since the main application of the method involves screening, qualitative performance of the method was evaluated for diluted extracts. In the case of 2-fold dilutions, the number of analytes that showed a rate of false negatives ≥10%, compared to C18 + hexane cleanup without dilution, increased from 15% to 30% and 7% to 15% at ½X and 1X levels, respectively. Therefore, dilution may be a good approach

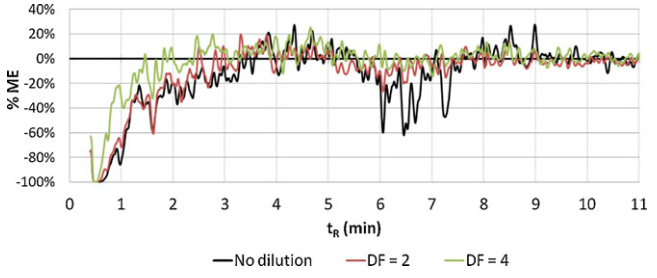

Fig. 5. Dilution factor (DF) vs. matrix effect (ME) for sulfamethoxypyridazine (m/z 281 → m/z 156).

to reduce MEs and improve ruggedness, but with the instrument we used, extract dilution reduced method performance at lower concentrations. Another important benefit of diluting the final extracts with water is that it reduces MeCN content of the injected samples and thereby significantly improves peak shapes of the early-eluting drugs.

3.6. Recoveries and repeatabilities

As previously discussed, some types of cleanup gave a higher removal of co-extractives and reduction of MEs than others. However, method performance is also needed to decide which of these cleanup sorbents should be used in the final method. Fig. 6 summarizes the recovery and precision results obtained for the different cleanup approaches. The use of Z-Sep$^+$ and Z-Sep + hexane gave significantly lower recoveries for many drugs, even though the precision was improved, as indicated by the percentage of analytes with reduced RSD_r. The percentage of analytes that gave 70–120% recoveries averaged ≈57% in cases of Z-Sep$^+$ and Z-Sep + hexane. None of the analytes significantly partitioned into hexane. This was confirmed by solvent-exchanging a portion of the hexane layer with mobile phase for analysis, in which no analytes were detected above trace levels.

Tetracyclines, fluoroquinolones, and macrolides were the 3 groups of drugs most retained by both Z-Sep$^+$ and Z-Sep + hexane. Additionally, Z-Sep + hexane strongly retained the β-lactams tested. Therefore, C18 + hexane was chosen as the best combination for the d-SPE format providing enough cleanup, lower MEs, recoveries between 70–120% and $RSD_r \leq 20\%$ for 75% of the analytes. Ultimately, the final method chosen remained as our method previously tested in beef kidney [7].

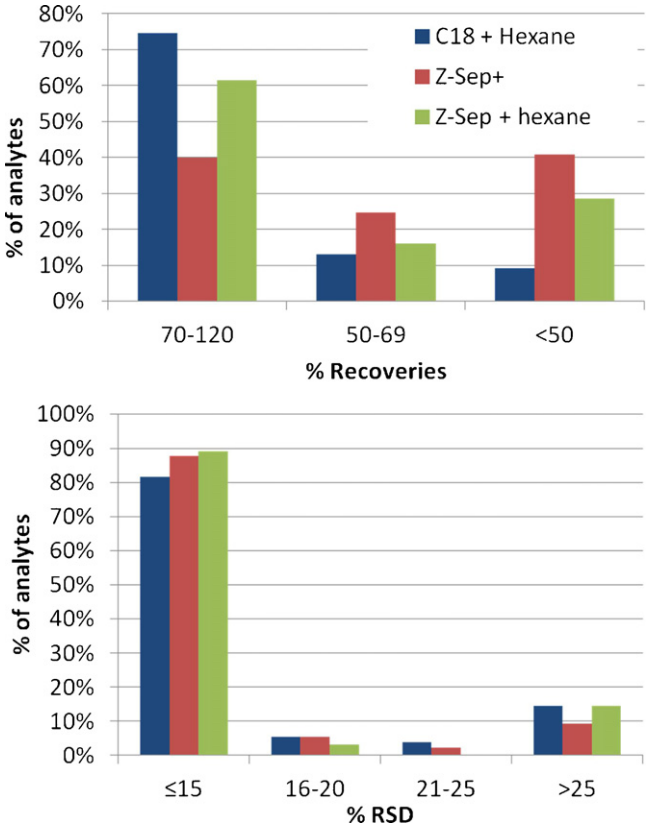

Fig. 6. Evaluation of method performance for 127 analytes testing C18 + hexane, Z-Sep$^+$ and Z-Sep + hexane cleanup approaches (n = 3) fortified at 2X spiking level for Set #1 and 200 ng/g for Set #2.

3.7. Analysis of incurred samples

Forty-four different bovine muscle samples, originating from carcasses of which the kidneys had screened positive using the 7-plate bioassay [7,36], were provided by FSIS. These animal tissues belonged to different subcategories: 8 beef cow, 6 bob veal, 4 bovine, 5 dairy cow, 9 formula-fed veal, 3 non-formula-fed veal, 3 heifer, 1 heavy calf and 5 steer. Each sample was screened using our final method, and sulfamerazine was identified near the tolerance level in 1 sample from formula-fed veal. Other veterinary drug residues present at levels < ½X were beta/dexamethasone (10 samples), chlortetracycline (6 samples), and 1 sample each of tetracycline, oxytetracycline, sulfapyridine, metronidazole, albendazole-2-amino-sulfone, and lincomycin. Most of the tissues with positive findings were formula-fed veal and bob veal. For development and validation, only the tissues that showed no positives (beef cow, dairy cow, heifer, bovine and steer) were used.

3.8. Qualitative screening

In this report, we make distinctions in terminology for qualitative analysis that were proposed by Lehotay et al. [37]. To meet FSIS screening criteria [7], a presumptive positive in LC–MS presents a chromatographic peak within ±5% of the reference standard's t_R for any of the ion transitions monitored. The signal (peak area) must be equal to or greater than the signal for the same ion transition obtained from a contemporaneously analyzed fortified sample at ½X tolerance level or other desired concentration (see Table 1). For drugs that have no tolerance, a threshold setting of signal-to-noise rate (S/N) > 3 can be set (in practice, we chose to use a peak area threshold setting of 100 to simplify this process). False negatives above the tolerance level are undesirable in regulatory screening. FSIS allows a rate of false positives ≤10% at the ½X level, but since UHPLC-MS/MS is more reproducible and selective than older LC–MS methods, we chose more stringent and practical criteria for ± difference in $t_R \leq 6$ s and acceptable rate of false positives ≤5%. In this way, the percentage of samples that would require re-analysis by the official regulatory method would be reduced.

In our analyses, the diverse range of cattle muscle samples showed no false positives due to matrix interferences. Thus, the ability of the screening method to distinguish the presence/absence of individual drugs is not an issue, and this approach works quite effectively for drugs without legal limits. It is more complicated to establish the optimal threshold signal level for drugs with a tolerance. The goal is to minimize the number of re-analyses that give results below the tolerances while mitigating the chances that actual violative samples will be missed. The arbitrary signal threshold from the fortified ½X tolerance samples can be further modified based on statistical results for drugs found in actual samples during routine usage of the method to better optimize this balancing act.

The validation study was done over the course of 4 and 6 days for Sets #1 and #2, respectively. Four different sources of C18 were used for both sets, and experiments on 2 additional days were performed using end-capped C18 for quantitation purposes of Set #2 analytes. The FSIS screening criteria were met for 113 of the 127 veterinary drugs (no false negatives and false positives ≤5%) at all spiking levels. Amoxicillin and ivermectin also met the screening criteria at the 2X spiking level (20 ng/g and 200 ng/g, respectively). In the case of the 3 earliest-eluting analytes (desacetyl cephapirin, florfenicol amine and 2-thiouracil), peak shapes and t_R were inconsistent, thus they did not meet the screening criteria. Furthermore, the method was not suitable for screening dipyrone, oxyphenylbutazone, phenylbutazone, and melengestrol acetate at all spiking levels in muscle. In the case of chloramphenicol, the main reason for unacceptable screening performance was due to the very low spiking level chosen (3 ng/g), and the method was still useful at higher concentrations. Four other veterinary drugs (tylosin, tulathromycin, triflupromazine and oxyclonazide) presented false negative rates < 2%, which technically did not meet the screening acceptability criteria, but the method might still be useful for analysis of those drugs in practice.

3.9. Qualitative identification

Three ion transitions were monitored for identification purposes, obtaining 3 ion ratios (2/1, 3/1, and 3/2). This approach gives a higher selectivity than monitoring only two ion transitions, which gives only one ion ratio (ion 2 vs. ion 1) for identification. All ion ratios were calculated by dividing the peak area for the ion of lesser average intensity by the more intense ion to yield ion ratios <100%. The identification criteria [7] defines a positive qualitative identification when the following conditions are met: (1) a chromatographic peak is present within ±0.1 min of the average t_R of the reference standard; (2) each ion transition monitored give very similar t_R and peak shapes; (3) the signal detected for the quantification ion (most intense ion) exceeds that of the contemporaneously analyzed matrix-matched calibration standard at ½X tolerance level (if no tolerance is set, S/N > 3 is achieved, or peak area threshold is exceeded); (4) the ion ratios for two transitions must be ≤|20%| absolute from the average ratio from the reference standards (or ion ratio ≤|10%| absolute for one ion transition); and (4) reagent and matrix blanks must not yield false positive identifications due to interferences, lab contamination, or carry-over.

An acceptability threshold of ≤10% false negatives for identification at each level tested was set for the purposes of this validation study, but even if the method does not meet that criterion at a particular spiking level and drug combination, that does not mean that the method is not useful to identify that drug for other purposes. All drug ion transitions can still be monitored at the given t_R in practice without affecting demonstrated quality of results for the other drugs. Examining multiple levels maximizes the inclusion of analytes for regulatory monitoring.

Table 2 gives the rates of false negatives in terms of identification for each analyte. The identification criteria were met for 98 drug analytes at all spiking levels, which grew to 111 analytes at the 1X level. Also, 74 analytes gave no false negatives at all, and only 11 analytes had a rate of false negatives >10% for all spiking levels. As stated in Section 3.8, the method was not suitable for desacetyl cephapirin, florfenicol amine, and 2-thiouracil due to peak shape and t_R inconsistencies. Additionally, poor results were obtained for amoxicillin, ivermectin, tulathromycin, ractopamine, and chloramphenicol (low spiking level). Curiously, low level interferences were present for cimaterol in muscle as we had also previously seen in kidney [7]. The second and third transitions did not meet the criteria at 3 ng/g, but acceptable results were obtained at 6 ng/g. Moreover, some of the muscle samples showed an interferant for hydroxyipronidazole's second ion transition, but it did not affect qualitative or quantitative results. No false positives occurred in any case, and in the cases of tetracycline and fenbendazole sulfone, 10% and 13% of the samples met t_R and ion ratio identification criteria, respectively; but neither exceeded the signal threshold at ½X tolerance levels.

3.10. Quantitative analysis

The main objective of this study was to demonstrate screening and identification qualitative aspects of the method for our application. However, quantification is a desirable additional benefit for as many drugs as possible. To begin, the QC and IS results were evaluated. Atrazine was added to the samples before filtration to isolate the filtration and analysis step, and two IS compounds,

Table 2
Qualitative (identification) and quantitative validation results for the final method in bovine muscle. False negatives > 10%, Rec < 70% and > 120%, average repeatability (RSD_r) > 20% and reproducibility (RSD_R) > 25% are given in bold text.

#	Analyte	1X spk (ng/g)	% False positives (n = 30–40)	False negatives				Avg %Rec. (n = 90–120)	Avg %RSD_r (n = 30–40)	%RSD_R (n = 90–120)
				½X spks (n = 30–40)	1X spks (n = 30–40)	2X spks (n = 30–40)	Overall (n = 90–120)			
1	Desacetyl Cephapirin	0.1	0	**100%**	**100%**	**100%**	**100%**	**0%**	N/A	N/A
2	Florfenicol Amine	0.3	0	**100%**	**100%**	**100%**	**100%**	**0%**	N/A	N/A
3	Sulfanilamide	0.1	0	**25%**	0%	0%	8%	81%	15%	13%
4	Amoxicillin	0.01	0	**87%**	**63%**	**45%**	**63%**	**66%**	**52%**	**49%**
5	Salbutamol	0.006	0	**30%**	**23%**	3%	**18%**	76%	17%	15%
6	Zilpaterol	0.012	0	**15%**	5%	3%	8%	**68%**	**26%**	23%
7	Cimaterol	0.006	0	**60%**	10%	0%	**23%**	87%	**23%**	21%
8	DCCD	1	0	0%	0%	0%	0%	**46%**	20%	16%
9	Lincomycin	0.1	0	0%	0%	0%	0%	76%	11%	10%
10	Sulfadiazine	0.1	0	0%	0%	0%	0%	81%	13%	13%
11	Ampicillin	0.01	0	0%	0%	0%	0%	70%	20%	19%
12	Desethylene Ciprofloxacin	0.05	0	5%	3%	0%	3%	**67%**	**30%**	**28%**
13	Sulfathiazole	0.1	0	0%	0%	0%	0%	79%	17%	16%
14	Sulfapyridine	0.05	0	**28%**	**30%**	8%	**22%**	80%	18%	16%
15	Norfloxacin	0.1	0	3%	5%	0%	3%	**67%**	**33%**	**30%**
16	Tulathromycin	0.1	0	**33%**	**25%**	**33%**	**30%**	**64%**	**47%**	**37%**
17	Oxytetracline	0.05	0	0%	0%	0%	0%	**41%**	20%	19%
18	Ciprofloxacin	1	0	10%	8%	5%	8%	**69%**	**29%**	**27%**
19	Ractopamine	0.002	0	**60%**	**38%**	**23%**	**40%**	79%	**32%**	**27%**
20	Sulfamerazine	0.10	0	0%	3%	3%	2%	82%	13%	12%
21	Danofloxacin	0.05	0	3%	3%	0%	2%	**68%**	**31%**	**29%**
22	Tetracyline	1	0[a]	0%	5%	0%	2%	**46%**	**25%**	21%
23	Enrofloxacin	0.05	0	0%	8%	5%	4%	**62%**	**30%**	**26%**
24	2-Quinoxalinecarboxylic Acid	0.03	0	**20%**	0%	0%	7%	77%	**25%**	23%
25	Sulfamethizole	0.1	0	0%	0%	0%	0%	77%	14%	13%
26	Sulfamethazine	0.1	0	5%	0%	3%	3%	82%	13%	13%
28	Cefazolin	0.1	0	3%	0%	0%	1%	83%	20%	19%
29	Sulfamethoxypyridazine	0.1	0	0%	3%	0%	1%	80%	13%	12%
30	Difloxacin	0.05	0	3%	0%	0%	1%	**66%**	14%	13%
31	Sarafloxacin	0.05	0	5%	0%	0%	2%	70%	17%	17%
32	Clenbuterol	0.006	0	8%	8%	3%	6%	82%	**24%**	23%
33	Pirlimycin	0.3	0	0%	0%	0%	0%	70%	19%	17%
34	Chlortetracycline	2	0	3%	0%	0%	1%	**40%**	12%	12%
35	Clindamycin	0.1	0	0%	0%	0%	0%	79%	13%	12%
36	Gamithromycin	0.1	0	3%	3%	8%	4%	78%	17%	14%
37	Sulfachloropyridazine	0.1	0	10%	10%	**20%**	**13%**	79%	11%	11%
38	Tilmicosin	0.1	0	0%	0%	0%	0%	78%	19%	18%
39	Sulfadoxine	0.1	0	0%	0%	0%	0%	82%	11%	10%
40	Sulfamethoxazole	0.1	0	0%	0%	0%	0%	80%	11%	11%
41	Sulfaethoxypyridazine	0.1	0	0%	0%	0%	0%	79%	12%	11%
42	Florfenicol	0.2	0	0%	0%	0%	0%	84%	15%	15%
43	Chloramphenicol	0.003	0	**93%**	**73%**	**53%**	**73%**	**124%**	**74%**	**67%**
44	Erythromycin	0.1	0	0%	0%	0%	0%	**68%**	**21%**	18%
45	Sulfadimethoxine	0.1	0	0%	0%	0%	0%	79%	9%	9%
46	Sulfaquinoxaline	0.1	0	**50%**	**50%**	**45%**	**48%**	77%	10%	9%
47	Prednisone	0.1	0	0%	0%	0%	0%	81%	11%	11%
48	Tylosin	0.2	0	0%	0%	0%	0%	76%	14%	12%
50	Penicillin G	0.050	0	0%	0%	0%	0%	74%	11%	11%
51	Beta/Dexa-methasone	0.1	0	0%	0%	0%	0%	81%	11%	11%
52	Sulfanitran	0.1	0	**20%**	**15%**	8%	**14%**	84%	14%	13%
53	Sulfabromomethazine	0.1	0	0%	0%	0%	0%	74%	12%	12%
54	Zeranol (α-Zearalanol)	0.006	0	**78%**	**40%**	10%	**43%**	81%	35%	**33%**
55	Oxacillin	0.1	0	0%	0%	0%	0%	76%	10%	9%
57	Cloxacillin	0.01	0	**23%**	3%	8%	**11%**	73%	16%	15%
58	Nafcillin	0.1	0	0%	0%	0%	0%	74%	9%	8%
59	Oxyphenylbutazone	0.1	0	5%	3%	**13%**	7%	**1491%**	**424%**	**93%**
60	Flunixin	0.025	0	0%	0%	**20%**	7%	81%	16%	12%
62	Dicloxacillin	0.1	0	0%	0%	0%	0%	76%	10%	9%
63	Phenylbutazone	0.1	0	**23%**	8%	5%	**12%**	**58%**	**115%**	**71%**
64	Melengesterol Acetate	0.02	0	**30%**	**25%**	**20%**	**25%**	**48%**	**58%**	**55%**
65	2-thiouracil	0.4	0	**100%**	**100%**	**100%**	**100%**	**0%**	**N/A**	**N/A**
66	2-mercapto-1-methylimidazole	0.2	0	0%	0%	0%	0%	73%	14%	14%
67	6-methyl-2-thiouracil	0.4	0	0%	0%	0%	0%	93%	17%	14%
68	Metronidazole-OH	0.01	0	**20%**	8%	0%	9%	93%	21%	20%
69	Dimetridazole-OH	0.05	0	0%	0%	0%	0%	92%	14%	14%
70	Metronidazole	0.01	0	10%	3%	0%	4%	97%	14%	13%
71	Ronidazole	0.01	0	5%	0%	0%	2%	92%	14%	13%

Table 2 (*Continued*)

#	Analyte	1X spk (ng/g)	% False positives (n = 30–40)	False negatives				Avg %Rec. (n = 90–120)	Avg %RSD_r (n = 30–40)	%RSD_R (n = 90–120)
				½X spks (n = 30–40)	1X spks (n = 30–40)	2X spks (n = 30–40)	Overall (n = 90–120)			
72	Dipyrone	0.2	0	**15%**	10%	5%	10%	**32%**	**161%**	**148%**
73	Levamisole	0.1	0	0%	0%	0%	0%	83%	11%	10%
74	Dimetridazole	0.01	0	3%	0%	0%	1%	87%	11%	10%
75	Albendazole 2-amino-sulfone	0.05	0	0%	0%	0%	0%	89%	18%	11%
76	5-Hydroxythiabendazole	0.1	0	0%	0%	0%	0%	71%	20%	18%
77	6-Propyl-2-thiouracil	0.05	0	3%	0%	0%	1%	92%	18%	16%
78	Thiabendazole	0.1	0	0%	0%	0%	0%	90%	10%	9%
79	2-Mercaptobenzimidazole	0.025	0	5%	0%	0%	2%	73%	**21%**	20%
80	Ipronidazole-OH	0.005	0	0%	0%	0%	0%	78%	13%	12%
81	Xylazine	0.01	0	**15%**	8%	0%	8%	71%	19%	18%
82	Orbifloxacin	0.05	0	0%	0%	0%	0%	83%	12%	11%
83	Morantel	0.1	0	**45%**	**25%**	**28%**	**33%**	**65%**	**23%**	**20%**
84	Azaperone	0.01	0	0%	0%	0%	0%	**63%**	**29%**	**26%**
85	6-Phenyl-2-thiouracil	0.4	0	0%	0%	0%	0%	83%	14%	12%
86	Clorsulon	0.1	0	0%	0%	0%	0%	86%	18%	17%
87	2-Amino-Mebendazole	0.01	0	**23%**	5%	0%	9%	82%	17%	15%
88	Albendazole sulfoxide	0.05	0	0%	0%	0%	0%	103%	14%	13%
89	Ipronidazole	0.001	0	8%	3%	0%	3%	78%	13%	12%
90	Carazolol	0.01	0	0%	0%	0%	0%	89%	17%	14%
91	2-Amino-Flubendazole	0.01	0	0%	0%	0%	0%	76%	16%	13%
92	Albendazole sulfone	0.05	0	0%	0%	0%	0%	88%	12%	11%
93	Doxycycline	0.1	0	0%	0%	0%	0%	**51%**	14%	12%
94	Oxfendazole	0.025	0	0%	0%	0%	0%	**150%**	**32%**	22%
95	Cambendazole	0.01	0	0%	0%	0%	0%	85%	13%	11%
96	Oxibendazole	0.01	0	0%	0%	0%	0%	82%	13%	12%
97	Nitroxynil	0.05	0	0%	0%	0%	0%	86%	11%	9%
98	Fenbendazole sulfone	1	0[b]	0%	0%	0%	0%	88%	10%	8%
99	Haloperidol	0.01	0	0%	0%	0%	0%	80%	18%	18%
100	Promethazine	0.01	0	0%	0%	0%	0%	**40%**	**25%**	22%
101	Bacitracin	0.5	0	0%	0%	0%	0%	**67%**	**21%**	19%
102	Acetopromazine	0.01	0	0%	0%	0%	0%	**51%**	**28%**	25%
103	Mebendazole	0.01	0	0%	0%	0%	0%	86%	14%	12%
104	Flubendazole	0.01	0	0%	0%	0%	0%	87%	15%	11%
105	Albendazole	0.05	0	0%	0%	0%	0%	**69%**	17%	15%
106	Propionylpromazine	0.01	0	0%	0%	0%	0%	**44%**	**30%**	**26%**
107	Chlorpromazine	0.01	0	0%	0%	0%	0%	**30%**	**37%**	**30%**
108	Ketoprofen	0.01	0	5%	0%	0%	2%	90%	20%	15%
109	Oleandamycin triacetate	0.15	0	0%	0%	0%	0%	74%	20%	19%
110	Triflupromazine	0.01	0	0%	0%	0%	0%	**35%**	**37%**	**30%**
111	Virginiamycin M1	0.1	0	0%	0%	0%	0%	81%	12%	12%
112	Haloxon	0.1	0	8%	3%	0%	3%	82%	**23%**	21%
113	Fenbendazole	0.4	0	0%	0%	0%	0%	74%	16%	14%
114	Diclofenac	0.2	0	**25%**	**25%**	**13%**	**21%**	73%	**24%**	20%
115	Triclabendazole sulfoxide	0.05	0	0%	0%	0%	0%	83%	14%	12%
116	Oxyclozanide	0.01	0	**20%**	8%	3%	10%	**48%**	**39%**	**30%**
117	Triclabendazole	0.05	0	0%	0%	0%	0%	82%	**23%**	22%
118	Tolfenamic acid	0.2	0	0%	0%	0%	0%	73%	**34%**	25%
119	Novobiocin	1	0	0%	0%	0%	0%	73%	18%	15%
120	Niclosamide	0.01	0	0%	0%	0%	0%	**59%**	18%	15%
121	Emamectin benzoate	0.01	0	0%	0%	0%	0%	76%	17%	16%
122	Bithionol	0.01	0	0%	0%	0%	0%	**44%**	**25%**	19%
123	Closantel	0.05	0	0%	0%	0%	0%	**60%**	**24%**	19%
124	Eprinomectin	0.1	0	0%	0%	0%	0%	78%	20%	18%
125	Abamectin	0.05	0	5%	0%	0%	2%	78%	**23%**	21%
126	Moxidectin	0.05	0	10%	3%	0%	4%	**68%**	**31%**	**27%**
127	Doramectin	0.1	0	**23%**	3%	8%	**11%**	78%	27%	25%
128	Rafoxanide	0.01	0	0%	0%	0%	0%	**39%**	**30%**	24%
129	Selamectin	0.2	0	**23%**	3%	10%	**12%**	76%	**24%**	20%
130	Ivermectin Na+	0.1	0	**35%**	**33%**	10%	**26%**	85%	**55%**	**50%**

[a] 10% of blanks met t_R and ion ratio criteria, but all concentrations < ½X tolerance.
[b] 13% of blanks met t_R and ion ratio criteria, but all concentrations < ½X tolerance.

sulfamethazine-$^{13}C_6$ and flunixin-d_3, were added along with the drug analytes prior to extraction. Satisfactory results were found for atrazine showing "recoveries" for Sets #1 and #2 of 104% and 102%, and reproducibility (RSD_R) of 14% and 5% (n = 160 and 120), respectively. Both IS exhibited 85% average recoveries with RSD_R <15%, except for sulfamethazine-$^{13}C_6$ in Set #1 which gave RSD_R > 25%. Since both IS were added from the same solution, pipetting errors could not be the cause of this higher variability. Because a new IS solution was made for the Day 4 experiment, the likely cause of this discrepancy was a mistake in preparation of the solution.

Among the factors considered studied in this validation process, the use of 4 different sources of C18 was evaluated. For the drugs in Set #1, no significant differences were observed among the various sources of C18. However, for Set #2, the use of

end-capped C18 enhanced the quantitative performance remarkably, showing an increase in acceptable recoveries (70–120%) from 55% to 75% of the analytes. Therefore, quantification validation of Set #2 drugs consisted of 3 days worth of data using end-capped C18 only.

All calibration curves were created using the LINEST function, for a linear trend forced through zero, in Excel except for nitroxynil, closantel, and triclabendazole sulfoxide (negative mode) and azaperone (positive mode) which followed binomial curves in their calibrations. Nevertheless, recovery calculations were done by direct comparison with the signal (peak area) of the matrix-matched standard at each corresponding level. Although IS were added to the samples, they were not used nor needed when calculating recoveries or compensating for MEs.

Table 2 shows the recovery and precision results for both sets of drugs. Recoveries falling outside the 70–120% range or with average $RSD_r > 20\%$ and $RSD_R > 25\%$ are given in bold font. Overall, the method demonstrated reasonably good quantitative performance as in previous MMM studies [7,19], with recoveries between 70–120% for 87 out of 127 analytes, and Rec < 50% for only 20 analytes. Moreover, 85 analytes gave $RSD_r \leq 20\%$ and 100 analytes gave $RSD_R \leq 25\%$.

The presence of interferants for cimaterol and hydroxyipronidazole did not affect their quantifier ions, thus good recoveries were obtained for both analytes. Certain groups of drugs showed worse performance than others. In the case of tetracyclines and fluoroquinolones, nearly all the drugs studied gave <70% recoveries. For tranquilizers and flukicides, ≈50% of the analytes showed acceptable results. In the future, the use of IS representative of these groups may be tested to correct for losses during extraction. Also, even though the post-column infusion for Set #2 considerably improved the sensitivities and qualitative results for anthelmintics, an increase in RSDs occurred for analytes with $t_R > 5$ min due to the flow and mobile phase composition variations introduced by the infusion technique in the system.

4. Conclusions

A rapid, reliable, and rugged MMM using UHPLC–MS/MS for analysis of >100 veterinary drugs in beef muscle using a rapid extraction followed by d-SPE cleanup with end-capped C18 + hexane partitioning was optimized and validated for use in monitoring by FSIS. This method was able to acceptably screen for 113 drugs, qualitatively identify 98, and quantify 87 out of 127 tested. A novel aspect of this method was the post-column infusion of mobile phase additives during the t_R of anthelmintic drugs to enhance their MS detection properties. In addition, MEs were carefully measured during method optimization using different cleanup options, chromatographic conditions, and dilution factors by a post-column infusion technique. Dilution of final extracts would be the best approach to reduce MEs, minimize instrument maintenance needs, and improve chromatography for the most polar analytes in the MMM, but the instrument would need to retain adequate sensitivity to continue to meet detection limit needs.

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